Research Article

Ultrastructural characteristics and immune profile of equine MSCs from fetal adnexa

Equine WJ and AM-MSCs in vitro features

Eleonora Iacono[a*], Luisa Pascucci[b], Barbara Rossi[a], Cinzia Bazzucchi[b], Aliai Lanci[a], Monica Ceccoli[a], Barbara Merlo[a]

[a] *Department of Veterinary Medical Sciences, University of Bologna, via Tolara di Sopra 50, 40064 Ozzano Emilia (BO), Italy.*

[b] *Department of Veterinary Medicine, University of Perugia, via San Costanzo 4, 06126 Perugia, Italy.*

*Corresponding author: Prof. E. Iacono, Department of Veterinary Medical Sciences, University of Bologna, via Tolara di Sopra 50, 40064 Ozzano Emilia (BO), Italy.

Tel.: +39 051 2097567

E-mail address: eleonora.iacono2@unibo.it

Abstract

Both in human and equine species, mesenchymal stem cells (MSCs) from amniotic membrane (AM) and Wharton's jelly (WJ), may be particularly useful for immediate use or in later stages of life, after cryopreservation in cell bank. The aim of this study was to compare equine AM- and WJ-MSCs *in vitro* features, that may be relevant for their clinical employment. MSCs were more easily isolated from WJ, even if MSCs derived from AM exhibited most rapid proliferation ($P<0.05$). Osteogenic and chondrogenic differentiation was most prominent in MSCs derived from WJ, as also suggested by the lower adhesion of AM cells, demonstrated by the greater volume of spheroids after hanging drop culture ($P<0.05$). Data obtained by PCR confirmed the immunosuppressive function of AM and WJ-MSCs and the presence of active genes specific for anti-inflammatory and angiogenic factors (IL-6, IL 8, IL-β1). For the first time, by means of transmission electron microscopy (TEM), we ascertained that equine WJ-MSCs constitutively contain a very impressive number of large vesicular structures, scattered throughout the cytoplasm and there was an abundant extracellular fibrillar matrix located in the intercellular spaces among WJ-MSCs. Results reveal that MSCs from different fetal tissues have different characteristics that may drive their therapeutic use. Data recorded in this study could be noteworthy for horses as well as for other mammalian species, including humans.

Keywords:

Mesenchymal stem cells, amniotic membrane, Wharton's jelly, equine, electron microscopy

Introduction

Mesenchymal stem cells (MSCs) are a population of multipotent stem cells and since their properties, MSCs offer a great chance for cell-based therapies and tissue engineering applications. Bone marrow (BM) is the common source of autologous MSCs for clinical applications in equine medicine. Alternatively, adipose tissue-derived MSCs can be used, since they have a higher proliferation potential (Iacono *et al.*, 2015a). Anyway, for both sources, an invasive procedure is required and there is a large variability in the cell yield related to the donor (Colleoni *et al.,* 2009). Furthermore, although bone marrow is the most widely investigated source of MSCs, these have limited potential in terms of in vitro proliferation capability (Guest *et al.,* 2010; Lange-Consiglio *et al.,* 2013), and do not appear to noticeably improve long-term functionality compared to those from extra-fetal tissues (Paris and Stout, 2010). Placental tissues and foetal fluids represent a source of cells for regenerative medicine, and are readily available and easily procured without invasive procedures. MSCs from foetal fluids and adnexa are defined as an intermediate between embryonic and adult SCs, due to the preservation of some characteristics typical of the primitive native layers (De Coppi *et al*., 2007). Among foetal adnexal tissues, the major sources of MSCs are amniotic membrane and Wharton's Jelly (Iacono *et al.,* 2015b). Althought, the increasing interest in using MSCs for regenerative medicine in horses, and the possibility to employ MSCs from perinatal tissue both for immediate use in newborns, both in later stages of life, after cryopreservation in cell bank, there is lacking of information on comparison between equine MSCs derived from AM and WJ were compared.

Usually, clinical treatments with MSCs are based on their transplantation but only a small percentage of the injected MSCs engraft successfully (Chimenti *et al*., 2010). Consistent with these findings, some studies recently showed that the regenerative ability of MSCs could be attributed to the production of molecules and mediators capable of activating the intrinsic repair processes in the damaged tissues. Different Authors, working on cardiac, renal, spinal cord and tendon regeneration, indicate that the beneficial effects of MSCs can be attributed to the activation of paracrine mechanisms enabling stimulation of endogenous stem cells. These cells are responsible for the bioactive soluble factors (lipids, growth factors, and cytokines) known to inhibit apoptosis and fibrosis, enhance angiogenesis, stimulate mitosis and/or differentiation of tissue-resident progenitor cells, and modulate the immune response (Yagi et *al.*, 2010; Liang *et al.*, 2014). Recently, the ability of equine adult MSCs to secrete numerous soluble mediators, implicated in the inhibition of T-cell proliferation, when stimulate with INF-gamma and TNG-alpha, was demonstrated (Carrade *et al.*, 2012; Kol *et al.*, 2013). However, to our knowledge, no studies are present on the immunophenotype profile, before in vitro stimulation, of equine WJ-MSCs and AM-MSCs, to better know their role in the immune response, angiogenesis, apoptosis, oxidation level and cell migration. Furthermore, in addition to soluble factors, recent findings indicate that extracellular vesicles are released from MSCs inside the CM and that these can be involved as important mediators in cell- to-cell communication (Pascucci *et al.*, 2014; Pascucci *et al.*, 2015). Microvesicles (MVs) have been categorized into exosomes (EXs), released from the endosomal compartment, and shedding vesicles (SVs), which bud directly from the cell membrane (Biancone *et al.*, 2012). MVs seem to be involved in a dynamic mutual paracrine communication between the embryonic and the maternal environment at the early stage of pre-implantation embryo development (Saadeldin *et al*., 2015). Recently, Lange-Consiglio *et al.* (2016) and Perrini *et al.* (2016) identified the presence and type of MVs secreted by equine AM-MSCs; the Authors also evaluated, in a preliminary study in vitro, the possible therapeutic implication of MVs in endometrial and tendon pathologies. Despite these studies on equine AM-MSCs, the recognized importance of WJ as an alternative source of MSCs both in equine and human medicine (Iacono *et al.*, 2012; Subramanian *et al.*, 2015) and despite a lot of data have been reported on these features of equine adult MSCs (Pascucci *et al.,* 2010; Maia *et al.,* 2013; Pascucci *et al*., 2014; Pascucci *et al.,* 2015), no studies are present on ultrastructural characteristics and MVs of equine WJ-MSCs. In this context, the aims of the present study were to analyze expression of stemness markers, immunophenotype, and ultrastructural features. In addition, we considered migration and adhesion ability of equine WJ-MSCs and AM-MSCs since both the migration ability, the expression of adhesion molecules and the homing to injured environments are important features of MSCs (Burk *et al.*, 2013; Kavanagh *et al.*, 2014).

Materials and Methods

Materials

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), and laboratory plastics from Sarstedt Inc. (Newton, NC, USA).

Animals

Samples were recovered from 13 Standardbred mares, housed at the Department of Veterinary Medical Sciences, University of Bologna, for attended delivery. Experimental procedures were approved by the Ethics Committee, University of Bologna (8134-X/10). The written consent was given by the owners to allow tissues recovery for research purposes.

Umbilical cord collection and WJ-MSCs isolation

Immediately after breaking the umbilical cord (UC), the part closest to the colt, characterized by an abundant amount of WJ, was severed. For avoiding mildew and bacterial contamination, samples were washed under flowing water for removing straw or feces debris.

Until processing, samples were stored in D-PBS (Dulbecco's Phosphatase Buffered Solution) containing penicillin (100 IU/ml) and streptomycin (100 mg/ml), at 4°C for at the latest 12 h. In the lab, before WJ enzymatic digestion, under a laminal flow hood, UCs were disinfected by immersing for few seconds in 70% ethanol and rinsed by repeated immersion in D-PBS. WJ was then isolated, weighed, minced finely (0.5 cm^2) by sterile scissors and cells were isolated as previously described (Iacono *et al.*, 2012). Briefly, WJ fragments were incubated in a 37 °C water bath for 1-2 h into a 50ml polypropylene tube, containing 1ml/1g sample of digestion solution (0.1% (w/v) collagenase type I (Gibco, Invitrogen Corporation, Carlsbad, California, USA), in D-PBS). The mixture was then filtered to separate the dispersed amnion cells from the tissue pieces and collagenase was inactivated by diluting 1:1 with D-PBS plus 10% (v/v) FBS (Gibco). Nucleated cells were pelleted at 470 g for 10 min. The supernatant was discarded, pellet was re-suspended in 5 ml of culture medium (Dulbecco's Modified Essential Medium (D-MEM)-F12 Glutamax® (Gibco) supplemented with 10% v/v FBS, 100 iu/ml penicillin and 100 µg/ml streptomycin) and spun at 470 g for 10 min to wash cells. This operation was repeated three times. After the last wash, cell pellet was re-suspended in 1 ml of culture medium and cell concentration was determined by haemocytometer.

Amnion collection and cells isolation

Allanto-amniotic membranes were obtained at pregnancy term, after vaginal delivery. Portions of allanto-amnion were washed under flowing water for removing straw or feces debris, stored at 4°C in D-PBS, added with antibiotics (100 iu/ml penicillin and 100 μg/ml streptomycin), and were processed within 12h. In the lab, before enzymatic digestion, under a laminal flow hood, samples were disinfected by immersing for few seconds in 70% ethanol and rinsed by repeated immersion in D-PBS. Then, AM was stripped from the overlying allantois, weighted and cut into small pieces (0.5 cm^2) by sterile scissors. Cells were then isolated as described above for WJ, by an enzymatic digestion.

Cell culture and proliferation assays

After isolation, primary cells derived from all recovered samples were plated in a 25 cm^2 flask in 5 ml of D-MEM-F12 Glutamax®, plus 10% v/v FBS and antibiotics. Cells were incubated in a 5% CO_2 humidified atmosphere at 38.5°C. At ~80-90% of confluence, they were dissociated by 0.25% trypsin, counted and plated at the concentration of $5x10^3$ cells/cm^2 as "Passage 1" (P1), and so on for the following passages. Calculation of cell-doubling time (DT) and cell-doubling numbers (CD) was carried out according to the formulae of (Rainaldi *et al.*, 1991):

$$CD = \ln(Nf/Ni)/\ln(2) \qquad (1)$$

$$DT = CT/CD \qquad (2)$$

where Nf is the final number of cells and Ni the initial number of cells.

Adhesion and Migration Assays

To define differences between WJ and AM-MSCs, spheroid formation and migration test were performed. Three replicates for each experiment were performed; all replicates were carried out at passage 3 of *in vitro* culture.

For adhesion assay, cells were cultured in 'hanging drops' (5.000 cells/drop of 25µl) for 24 h. Images were acquired by a Nikon Eclipse TE 2000-U microscope. Spheroid areas were determined using ImageJ software (imagej.nih.gov/ij/). Starting from the binary masks obtained by Image J, the volume of each spheroid was computed using ReViSP (sourceforge.net/projects/revisp) (Bellotti *et al.*, 2016), a software specifically designed to accurately estimate the volume of spheroids and to render an image of their 3D surface.

To assess cell migration potential, a scratch assay (also known as Wound-Healing assay) was carried out, as previously described (Liang *et al.*, 2007). Briefly, at 80–90 % confluence the cell monolayer was scraped using a p1000 pipet tip. After washing twice with D-PBS, the dish was incubated for 24 h at 38.5 °C and 5 % CO_2 in a humidified atmosphere. Images were acquired both immediately after the tip-scratch (time 0; T0) and after the incubation period (last time point or time 1; T1), and the distances of each scratch closure were calculated by ImageJ software. The migration percentages were calculated using the following formula (Rossi *et al.*, 2014):

$$[(\text{distance at T0} - \text{distance at T1}) * 100] / \text{distance at T0}$$

In vitro differentiation

In vitro differentiation potential of cells towards osteogenic, adipogenic and chondrogenic lineages was studied. Cells ($5x10^3$ cells ⁄cm^2) were cultured under specific induction media (Table 1). As negative control, an equal number of cells was cultured in expansion medium. *In vitro* differentiation potential was assessed at passage 3 of culture in two replicates for 3 samples from each lineage. To cytologically evaluate differentiation, cells were fixed with 10% formalin at room temperature (RT) and stained with Oil Red O, Alcian Blue and Von Kossa for adipogenic, chondrogenic and osteogenic induction, respectively. Quantitative analysis of *in vitro* differentiation was performed by Image J.

Immunocytochemistry (ICC)

Cells, derived from 3 AM and WJ samples, at P3, were cultured on ICC slides, until confluence. They were then fixed with 4 % paraphormaldehyde (20 min at RT) and then washed in phosphate buffer (PB). Cells were blocked in goat serum (10 %) for 1 h and incubated overnight with primary antibodies (Table 2); the day after, they were washed in PB2 (PB + 0.2 % BSA + 0.05 % saponin) and incubated with anti-mouse- or anti-rabbit-FITC conjugated secondary antibodies for 1 h. Nuclei were then labelled with Hoechst 33342. The excess of secondary antibody and Hoechst were removed by 3 washes with PB2. Images were obtained with a Nikon Eclipse E400 microscope, using the software Nikon NIS-Elements.

Molecular Characterization

To evaluate pluripotency potential of the two types of equine cells, PCR for the pluripotency genes OCT4, NANOG and SOX2 was performed. Gene expression was tested on equine blastocysts, as positive control. To test cell stemness and immunoproperty, the following set

of genes was evaluated: CD45, CD 34, CD90, CD73, MHC-I, MHC-II, IL-β1, IL-4, IL-6, IL-8, INF-γ, TNF-α. Primers were tested on activated equine lymphocyte. The specific set of primers used is listed in Table 3. All tests were carried out on 100×10^3 cells, derived from AM and WJ of three different mares. Experiments were performed at passage 3 of culture.
Cells were snap-frozen and RNA was extracted using Nucleo Spin® RNA kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. cDNAs were synthesized by RevertAid RT Kit (ThermoFisher Scientific, Waltham, Massachusetts, USA) and used directly in PCR reactions, following the instructions of Maxima Hot Start PCR Master Mix (2X) (ThermoFisher Scientific, Waltham, Massachusetts, USA). PCR products were visualized with ethidium bromide on a 2% agarose gel.

Transmission Electron Microscopy (TEM)

Ultrastructural examination was performed on AM (n=3) and WJ-MSCs (n=3) at P3. The analysis was performed on three replicates. After detaching cells, the pellet was fixed with 2.5% glutaraldehyde in 0.1 M PB, pH 7.3, for 1 h, at RT. Cells were then washed twice in PB and post-fixed with buffered 2% osmium tetroxide for 1 h, at RT. They were finally dehydrated in a graded ethanol-propylen oxide series, pre-infiltrated and embedded in Epon 812. Ultrathin sections (90 nm) were mounted on 200-mesh copper grids, stained with uranyl acetate and lead citrate, and examined by a Philips EM 208 microscope, equipped with a digital camera (Center for Electron Microscopy, CUME, University of Perugia).

Statistical Analysis

Harvested WJ and AM (grams), CDs, DTs and percentages of migration are expressed as mean ± standard deviation (SD). Statistical analyses were performed using IBM SPSS Statistics 21 (IBM Corporation, Armonk, New York, USA). Data were analysed, for normal distribution, using a Shapiro-Wilk test, then using one-way ANOVA or a Student's t-test (CDs and DTs). The 3D spheroid volumes and mean grey intensity of differentiated cells were compared using Mann-Whithney's U-test, due to their non-normal distribution. Significance was assessed for $P<0.05$.

Results

Cellular Growth

As soon as after foals' birth and immediately after foal detachment, UC (length ~15 cm) and AM samples were recovered. The mean weight of recovered jelly and AM were 5.22±3.34 g and 15.60±5.23 g, respectively. Adherent mononuclear cells, characterized by elongated fibroblast-like morphology were isolated in 13/13 (100%) WJ samples and in 9/13 (69.2%) AM samples. Undifferentiated cells of both lines were passaged up to seven times; no changes in cell morphology was observed throughout the culture period. DTs assay showed that AM and WJ-MSCs were able to divide for an extensive period *in vitro*. During P0 to P7, AM-MSCs showed a mean DT of 1.49±0.34 days/CD, significantly lower than that recorded for WJ-MSCs (1.71±0.65 days/CD; $P<0.05$). No statistically significant differences were found in DTs among earlier culture passages in both cell lines ($P>0.05$). However, AM-MSCs start to grow more slowly by P6, a sign of cellular aging; on the contrary, WJ-MSCs, despite an higher DT, get older later, starting from P7. This is confirmed by significantly higher DTs compared to the earliest steps ($P<0.05$). By P7, total WJ and AM-MSCs cell doublings were similar (36.57±0.76 vs 37.05±0.59; $P>0.05$; Fig.1).

Adhesion and Migration Assays

Both AM and WJ cells formed spheroids when cultured in hanging drops. Average areas and volume of the spheroids formed by WJ-MSCs were significantly smaller than from AM-MSCs ($P<0.05$; Fig.2). Average percentage of migration, observed by scratch test, was statistically similar between cell lines (AM-MSCs vs WJ-MSCs: 34.14±4.51% vs 38.20±2.88%; $P>0.05$; Fig. 2).

In vitro Differentiation

Both cell lines were able to differentiate toward osteogenic, chondrogenic and adipogenic direction (Fig.3). However, WJ-MSCs showed a greater chondrogenic and osteogenic potential ($P<0.05$), characterized by a greater accumulation of extra-cellular mucosubstances and calcium deposits, as showed by Alcian Blu (Fig. 3A) and Von Kossa (Fig.3B) stains respectively.

Immunostaining and PCR analysis

Immunostaining results are showed in Figure 4. Amniotic membrane and WJ MSCs clearly expressed mesenchymal marker, N-Cadherin, and the mesodermal marker alpha-SMA. On the contrary, they did not express pan-cytokeratin and E-Cadherin.

PCR results are reported in Table 4; positive expression are also showed in Figure 5. Both cell populations expressed MSC-associated markers (CD90, CD73), while were negative for an hematopoietic marker (CD45), at P3 of *in vitro* culture. On the contrary, the haematopoietic marker CD34 was registered for either population. Both WJ-MSCs and AMSCs lacked MHC-I and MHC-II expression. Regarding embryonic markers, WJ-MSCs expressed OCT-4, while AM-MSCs were weakly positive for this marker; both cell populations lacked Nanog and Sox2. About their immune-phenotype, both WJ-MSCs and AMSCs lacked MHC-I, MHC-II, INF- γ, TNF-α and IL-4 expression. Cell were instead positive for IL-6 and IL- β1. WJ-MSCs expressed IL-8 marker, while a weak expression was showed by AM-MSCs.

TEM

At low magnification, cells of both samples were quite small and uniform in size (diameter range: 10-15 μm; Fig. 6A; Fig. 7A). AM-MSCs appeared generally well dissociated, while WJ MSCs were frequently tightly adherent each other to form wide aggregates (Fig. 6A and B). Golgi complex was particularly well developed; it occupied a juxta-nuclear position and exhibited flattened cisternae, transport vesicles, and heterogeneous sized secreting granules. Some of them were very large and enclosed fine granular material (Fig. 6C and D). RER showed linear flat profiles and dilated cisternae (Fig. 6E and F). In both samples, the most interesting ultrastructural feature was represented by the very impressive number of large vesicular structures, up to 2 μm in diameter, scattered throughout the cytoplasm (Fig. 6G and H). They showed a variety of appearances and ranged from multivesicular bodies (MVB) (Fig. 7A and B) comprising intralumenal nanovesicles of different sizes (30-500 nm), to endolysosomes and autophagic vacuoles. They were particularly abundant in WJ-MSCs. The occurrence of membrane vesicles shedding from cell surface was observed in both samples. They ranged in size from 100 nm to 500 nm and included electron-lucent, as well as moderately electron-dense vesicles isolated or aggregated nearby the cells (Fig. 7C and D).

Complex extracellular vesicles measuring 500nm-1μm and containing packed nanovesicles, frequently budded from the cell surface or were detected in the intercellular space (Fig. 7E and F). Tunneling nanotubes were occasionally observed in both samples suggesting that this may be an additional mechanism of crosstalk between MSCs (Fig. 7G and H). The most noteworthy difference between AM-MSCs and WJ-MSCs was the presence of an abundant extracellular fibrillar matrix (EFM) located in the intercellular spaces among WJ-MSCs (Fig.

8A-C). It was composed of a finely granular and moderately electron-dense ground substance populated by a loosely arranged network of reticular fibrils. These were uniformly thin and tend to run parallel to the cell surface. Abundant vesicles were entrapped among the fibrils (Fig. 8C). The intercellular spaces were devoid of collagen fibrils.

Discussion

AM-MSCs and WJ-MSCs are the focus of great interest in human and veterinary regenerative medicine for their in vitro multilineage differentiation potential, their great in vitro expansion (Iacono *et al.,* 2012; Lange-Consiglio *et al.*, 2013). In the present study, for the first time in equine species, proliferation, migration, spheroids formation, trilineage differentiation capacity, expression of stemness markers, immunophenotype and ultrastructural features of MSCs derived from WJ and AM were compared. From both tissues, cells with mesenchymal morphology were isolated. However, as recently reported in human (Subramanian *et al.*, 2015), in the present study, MSCs were isolated from all samples by collagenase digestion technique only for WJ. No other reports exist on the successful isolation rate from equine WJ and AM. Despite the lower isolation rate, AM-MSCs showed a higher proliferation rate compared to WJ-MSCs. As in human (Pasquinelli *et al.*, 2007), in both cell types, TEM examination revealed an highly metabolic and synthetic nature, demonstrated by euchromatic nucleus, prominent nucleoli, abundant nuclear pores as well as by well-developed RER and Golgi complex. Furthermore, the higher DTs were unrelated with total cell doubling number, because AM-MSCs began to grow old earlier, as registered by a higher DT at P6 of in vitro culture, confirming the proliferative nature of WJ-MSCs. Beyond the growth curve, migration ability is an important feature of MSCs because of its fundamental significance for systemic application (Li *et al.*, 2009; Burk *et al.*, 2013). No differences were found between WJ-MSCs and AM-MSCs in migration ability. Since the adhesion capability is related and enhanced to differentiation potential (Pasquinelli *et al.*, 2007; Wang *et al.*, 2009; Kavanagh *et al.*, 2014), in the present study spheroid formation *in vitro* was assessed using the hanging drop method. Cell derived from WJ showed a higher adhesion ability, forming smaller spheroids, as determined by ReVisp. In the present study, the analysis of differentiated cells by Image J showed a higher WJ-MSCs chondrogenic and osteogenic potential. Our results confirmed data recently registered with human WJ-MSCs and AM-MSCs cell (Subramanian *et al.* 2015), in fact also equine WJ-MSCs, exposed to osteocyte and chondrocyte differentiation media, showed the highest number of Von Kossa stained cells, greatest staining intensity of nodules and higher number of cells positive for Alcian Blue compared to cells from AM (Subramanian *et al.*, 2015). Besides to differentiation ability, the equine fetal adnexa derived MSCs demonstrate the characteristics defined by the International Society for Cellular Therapy criteria (Dominici *et al.*, 2006), except for the CD34. CD34 is predominantly regarded as a marker of hematopoietic stem cells (HSC) and hematopoietic progenitor cells. Accumulating evidence demonstrates CD34 expression on several other cell types, including embryonic stem cell derived MSC (Kopher *et al.*, 2010) and multipotent mesenchymal stromal cells (MSC) (Nielsen and McNagny, 2008). In many cases, CD34 indicate a distinct subset of cells with enhanced progenitor activity (Sidney *et al.*, 2014). The expression of CD34 by equine cells might constitute evidence of their potenciality. Moreover, as intermediate between adult and embryonic cells, equine WJ and AM-MSCs express OCT-4, a marker for pluripotent stem cells. However, as previously reported in human (Subramanian *et al.*, 2015), also in equine, the expression level of OCT-4 seems to be lower for cells from AM compared to WJ. This finding, coupled with greater differentiation ability, could be related to the middle position of WJ between blastocyst and adult. The stem cells isolated from the WJ probably start to lose their embryonic pluripotency tumorigenic characteristics and start to acquire multipotent non-tumorigenic MSC characteristics with progressive development. This feature would help cells from the WJ to differentiate into specific lineages more easily both in vitro and during cell-based therapy and allow higher reprogramming efficiency to the embryonic state because of an immature phenotype (Pera *et al*., 2009). In human cells derived from WJ, the telomerase levels remained high throughout serial culture compared to AM-MSCs suggesting that they retain their primitive characteristics in culture for long periods of time (Subramanian *et al.*, 2015). In equine species further studies are needed to verify this condition.

Due to the importance of MSCs for their immune response and their ability to suppress T-cells (Carrade *et al.*, 2012), in the present study, anti-inflammatory and pro-inflammatory factors produced both by WJ-MSCs and AM-MSCs were investigated for the first time in the horse. One of the most important cytokines of the acute phase reaction is TNF-α, while IL-4 is a cytokine involved in allergic inflammation. Different from that observed in human cells, equine WJ and AM-MSCs do not express these markers, neither INF-γ when they are not stimulate in vitro by the presence of INF. Confirming their reduced immunogenicity, both cell lines were negative for MHC-I and MHC-II. On the contrary, both cell lines expressed, on their cDNA, IL-1β, IL-6 and IL-8; this cytokines are important mediator of the inflammatory response, involved in a variety of cellular activities, including cell proliferation, differentiation, apoptosis, chemotaxis, angiogenesis and hematopoiesis (Lamalice *et al.*, 2007). Data registered in this study confirmed those already reported in human WJ-MSCs (Dominici *et al.*, 2006; Choi *et al.*, 2013) and AM-MSCs (Yazdanpanah *et al.*, 2015). These factors are involved in the complex interaction between MSCs and the tissue microenvironment as well as in the production of membrane vesicles, containing molecules such as short peptides, proteins, lipids, and various forms of RNAs (György *et al.*, 2011). As previously observed in adult equine cells (Pascucci *et al.*, 2014), the great number of MVB that, contained intralumenal vesicles maturing from their internal membrane, may be interpreted as the ability of both cell types to produce a huge variety of “secreting” molecules enclosed inside vesicles of different types that are released in the extracellular milieu. Maybe hypothesized, in addition, that the several other vesicular structures observed by TEM represent a mechanism to efficiently recycle cell constituents by autophagy. The intense proliferating and metabolic activity, in fact, makes it necessary to constantly renew sub-cellular components, especially membrane fractions. The main difference between AM-MSCs and WJ-MSCs attained the presence of an abundant extracellular fibrillar matrix in the intercellular spaces among WJ-MSCs; it probably determines a tight intercellular adhesion even after trypsin treatment and is responsible for the observation of cell aggregates at TEM analysis. It is well known that these cells, in vivo, are immersed in a mucoid connective matrix. It seems evident that WJ-MSC isolation and cultivation in vitro does not affect their ability to produce extracellular matrix.

Conclusion

It has emerged from the present study that cells isolated from different fetal origin matrices exhibit different morphological, molecular and differentiation potential. Equine WJ could be considered as a viable source for MSCs with reliable migration and differentiation capacities, and it is therefore a convenient cell source for autologous or allogeneic regenerative therapies. Although the molecular content and functional activities of EVs produced by WJ and AM-MSCs remain to be characterized, the results of the present study indicated that MSCs from equine fetal adnexa are able to constitutively produce EVs that may be partly responsible for their paracrine activity. Further investigation are needed to find the best protocols for isolation and *in vitro* differentiation for AM-MSCs. Moreover, additional *in vivo* tests are needed to confirm our *in vitro* findings.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Acknowledgments

The authors wish to thank Prof. Carolina Castagnetti (Head of Equine Clinical Service, DIMEVET, University of Bologna, Italy) for the agreement to the sample collection. They wish also to thank all of the technical staff and veterinarians at Equine Perinatology and Reproduction Unit, DIMEVET, University of Bologna, for their assistance with this project.

References

Bellotti C, Duchi S, Bevilacqua A, Lucarelli E & Piccinini F 2016 Long term morphological characterization of mesenchymal stromal cells 3D spheroids built with a rapid method based on entry-level equipment. *Cytotechnology* **68** 2479–2490.

Biancone L, Bruno S, Deregibus MC, Tetta C & Camussi G 2012 Therapeutic potential of mesenchymal stem cell-derived microvesicles. *Nephrology Dialisis Transplantation* 27 3037–3042.

Burk J, Ribitsch I, Gittel C, Juelke H, Kasper C, Staszyk C & Brehm W 2013 Growth and differentiation characteristics of equine mesenchymal stromal cells derived from different sources. *The Veterinary Journal* **195** 98–106.

Carrade DD, **Lame MW, Kent MS, Clark KC, Walker NJ & Borjesson DL** 2012 Comparative analysis of the immunomodulatory properties of Equine Adult-Derived Mesenchymal Stem Cells. *Cell Medicine* **4** 1-11.

Castagnetti C, Mariella J, Pirrone A, Cinotti S, Mari G & Peli A 2012 Expression of interleukin-1 β, interleukin-8, and interferon-γ in blood samples obtained from healthy and sick neonatal foals. *American Journal of Veterinary Research* **73** 1418–1427.

Chimenti I, Smith RR, Li TS, Gerstenblith G, Messina E, Giacomello A & Marbán E 2016 Relative roles of direct regeneration versus paracrine effects of human cardiosphere-derived cells transplanted into infarcted mice. *Circulation Research* **106** 971–980.

Choi M, Lee H, Naidansaren P, Kim H, Cha J, Ahn H, Yang P, Shin J & Joe Y 2013 Proangiogenic features of Wharton's jelly-derived mesenchymal stromal/stem cells and their ability to form functional vessels. *The International Journal of Biochemistry and Cell Biology* **45** 560–570.

Colleoni S, Bottani E, Tessaro I, Mari G, Merlo B, Romagnoli N, Spadari A, Galli C & Lazzari G 2009 Isolation, growth and differentiation of equine mesenchymal stem cells:

effect of door, source, amount of tissue and supplementation with basic fibroblast growth factor. *Veterinary Research Communication* **33** 811-821.

Corradetti B, Lange-Consiglio A, Barucca M, Cremonesi F & Bizzarro D 2011 Size-sieved subpopulations of mesenchymal stem cells from intervascular and perivascular equine umbilical cord matrix. *Cell Proliferation* **44** 330–342.

De Coppi P, Bartsch G, Siddiqui MM, Xu T, Santos CC, Perin L, Mostoslavsky G, Serre AC, Snyder EY, Yoo JJ, Furth ME, Soker S & Atala A 2007 Isolation of amniotic stem cell lines with potential for therapy. *Nature Biotechnology* **25** 100–106.

Desmarais J, Demers S, Suzuki JJ, Laflamme S, Vincent P, Laverty S & Smith L 2011 Trophoblast stem cell marker gene expression in inner cell mass-derived cells from parthenogenetic equine embryos. *Reproduction (Cambridge, England)* **141** 321–332.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D & Horwitz E 2006 Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8** 315–317.

Guest DJ, Smith MR & Allen WR 2010 Equine embryonic stem-like cells and mesenchymal stromal cells have different survival rates and migration patterns following their injection into damaged superficial digital flexor tendon. *The Equine Veterinary Journal* **42** 636–642.

György B, Szabò T, Pàsztòi M, Pàl Z, Mijàk P, Aradi B, Làszlò V, Pàllinger E, Pap E, Kittel A *et al.* 2011 Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cellular and Molecular Life Sciences* **68** 2667–2688.

Iacono E, Brunori L, Pirrone A, Pagliaro PP, Ricci F, Tazzari PL & Merlo B 2012 Isolation, characterization and differentiation of mesenchymal stem cells from amniotic fluid, umbilical cord blood and Wharton's jelly in the horse. *Reproduction (Cambridge, England)* **143** 455–468.

Iacono E, Merlo B, Romagnoli N, Rossi B, Ricci F & Spadari A 2015a Equine bone marrow and adipose tissue mesenchymal stem cells: Cytofluorimetric characterization, in vitro differetiation and clinical application. *Journal of Equine Veterinary Sciences* **35** 130-140.

Iacono E, Rossi B & Merlo B 2015b Stem cells from foetal adnexa and fluid in domestic animals: an update on their features and clinical application. *Reproduction in Domestic Animals* **50** 353-364.

Jischa S, Walter I, Nowotny N, Palm F, Budik S, Kolodziejek J & Aurich C 2008 Uterine involution and endometrial function in postpartum pony mares. *American Journal of Veterinary Research* **69** 1525–1534.

Kavanagh D, Robinson J & Kalia N 2014 Mesenchymal stem cell priming: fine-tuning adhesion and function. *Stem Cell Reviews* **10** 587–599.

Kol A, Walker NJ, Galuppo LD, Clark KC, Buerchler S, Bernanke A & Borjesson DL 2013 Autologous point-of-care cellular therapies variably induce **equine**mesenchymal stem cell migration, proliferation and cytokine expression. *The Equine Veterinary Journal* **45** 193-198.

Kopher RA, Penchev VR, Islam MS, Hill KL, Khosla S & Kaufman DS 2010 Human embryonic stem cell-derived CD34+ cells function as MSC progenitor cells. *Bone* **47** 718–728.

Lamalice L, Le Boeuf F & Huot J 2007 Endothelial cell migration during angiogenesis. *Circulation Research* **100** 782–794.

Lange-Consiglio A, Corradetti B, Bizzaro D, Magatti M, Ressel L, Tassan S & Parolini O, Cremonesi F 2012 Characterization and potential applications of progenitor cells isolated from horse amniotic membrane. *Journal Tissue Engineering Regenerative Medicine* **6** 622-635.

Lange-Consiglio A, Corradetti B, Meucci A, Bizzaro D & Cremonesi F 2013 Characteristics of equine mesenchymal stem cells derived from amnion and bone marrow: in vitro proliferative and multilineage potential assessment. *The Equine Veterinary Journal* **45** 737–744.

Lange-Consiglio A, Perrini C, Tasquier R, Deregibus M, Camussi G, Pascucci L, Marini M, Corradetti B, Bizzarro D, De Vita B *et al.* 2016 Equine Amniotic Microvesicles and Their Anti-Inflammatory Potential in a Tenocyte Model In Vitro. *Stem Cells Development* **25** 610–621.

Li G, Zhang X, Wang H, Wang X, Meng C, Chan C, Yew D, Tsang K, Li K, Tsai S *et al.* 2009 Comparative proteomic analysis of mesenchymal stem cells derived from human bone marrow, umbilical cord and placenta: implication in the migration. *Proteomics* **9** 20–30.

Liang CC, Park AY & Guan JL 2007 In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nature Protocols* **2** 329–333.

Liang X, Ding Y, Zhang Y, Tse H & Lian Q 2014 Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives. *Cell Transplantation* **23** 1045–1059.

Maia L, Landim-Alvarenga FC, Da Mota LS, De Assis Golim M, Laufer-Amorim R, De Vita B, Barberini DJ, Listoni AJ, De Moraes CN, Heckler MC & Amorim RM 2013 Immunophenotypic, immunocytochemistry, ultrastructural, and cytogenetic characterization of mesenchymal stem cells from equine bone marrow. *Microsc Res Tech* **76** 618-624.

Mizuno H & Hyakusoku H 2003 Mesengenic potential and future clinical perspective of human processed lipoaspirate cells. *Journal of Nippon Medical School* **70** 300–306.

Mohanty N, Gulati B, Kumar R, Gera S, Kumar P, Somasundaram R & Kumar S 2014 Immunophenotypic characterization and tenogenic differentiation of mesenchymal stromal cells isolated from equine umbilical cord blood. *In Vitro Cellular & Developmental Biology - Animal* **50** 538–548.

Nielsen J & McNagny K 2008 Novel functions of the CD34 family. *Journal of Cell Science* **121** 3683–3692.

Paris DB & Stout TA 2010 Equine embryos and embryonic stem cells: defining reliable markers of pluripotency. *Theriogenology* **74** 516–524.

Pascucci L, Mercati F, Marini C, Ceccarelli P, Dall'Aglio C, Pedini V & Gargiulo AM 2010 Ultrastructural morphology of equine adipose-derived mesenchymal stem cells. *Histol Histopathol* **25** 1277-1285.

Pascucci L, Alessandri G, Dall'Aglio C, Mercati F, Cloliolo P, Bazzucchi C, Dante S, Petrini S, Curina G & Ceccarelli P 2014 Membrane vesicles mediate pro-angiogenic activity of equine adipose-derived mesenchymal stromal cells. *The Veterinary Journal* **202** 361–366.

Pascucci L, Dall'Aglio C, Bazzucchi C, Mercati F, Mancini M, Pessina A, Alessandri G, Giammaroli M, Dante S, Brunati G *et al.* 2015 Horse adipose-derived mesenchymal stromal cells constitutively produce membrane vesicles: a morphological study. **30** 549–557.

Pasquinelli G, Tazzari PL, Ricci F, Vaselli C, Buzzi M, Conte R, Orrico C, Foroni L, Stella A, Alviano F *et al.* 2007 Ultrastructural characteristics of human mesenchymal stromal (stem) cells derived from bone marrow and term placenta. *Ultrastructural Pathology* **31** 23–31.

Pera MF 2009 Stem cells: Low-risk reprogramming. *Nature* **458** 715–716.

Perrini C, Strillacci MG, Bagnato A, Esposti P, Marini MG, Corradetti B, Bizzaro D, Idda A, Ledda S, Capra E, Pizzi F, Lange-Consiglio A & Cremonesi F (2016) Microvesicles secreted from equine amniotic-derived cells and their potential role in reducing inflammation in endometrial cells in an in-vitro model. *Stem Cell Research and Therapy* **18** 169.

Rainaldi G, Pinto B, Piras A, Vatteroni L, Simi S & Citti L 1991 Reduction of proliferative heterogeneity of CHEF18 Chinese hamster cell line during the progression toward tumorigenicity. *In Vitro Cellular & Developmental Biology 27A* **27** 949–952.

Rossi B, Merlo B, Colleoni S, Iacono E, Tazzari PL, Ricci F, Lazzari G & Galli C 2014 Isolation and in vitro characterization of bovine amniotic fluid derived stem cells at different trimesters of pregnancy. *Stem Cell Reviews* **10** 712–724.

Saadeldin IM, Oh HJ & Lee BC 2015 Embryonic-maternal cross-talk via exosomes: potential implications. *Stem Cells Cloning* **8** 103–107.

Sidney LE, Branch MJ, Dunphy SE, Dua HS & Hopkinson A 2014 Concise Review: Evidence for CD34 as a Common Marker for Diverse Progenitors. *Stem Cells (Dayton, Ohio)* **32** 1380–1389.

Subramanian A, Fong C, Biswas A & Bongso A 2015 Comparative Characterization of Cells from the Various Compartments of the Human Umbilical Cord Shows that the Wharton's Jelly Compartment Provides the Best Source of Clinically Utilizable Mesenchymal Stem Cells. *Plos One* **10** e0127992.

Visser M & Pollitt C 2011 Lamellar leukocyte infiltration and involvement of IL-6 during oligofructose-induced equine laminitis development. *Veterinary Immunology and Immunopathology* **144** 120–128.

Wang W, Itaka K, Ohba S, Nishiyama N, Chung U, Yamasaki Y & Kataoka K 2009 3D spheroid culture system on micropatterned substrates for improved differentiation efficiency of multipotent mesenchymal stem cells. *Biomaterials* **30** 2705–2715.

Yagi H, Soto-Gutierrez A, Parekkadan B, Kitagawa Y, Tompkins RG, Kobayashi N & Yarmush ML 2010 Mesenchymal stem cells: mechanisms of immunomodulation and homing. *Cell Transplant* **19** 667–679.

Yazdanpanah G, Paeini-Vayghan G, Asadi S & Niknejad H 2015 The effects of cryopreservation on angiogenesis modulation activity of human amniotic membrane. *Cryobiology* **71** 413–418.